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# BOTULINUM AND TETANUS NEUROTOXINS

*Neurotransmission and  
Biomedical Aspects*

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## CURRENT CONCEPTS ON THE MECHANISM OF ACTION OF CLOSTRIDIAL NEUROTOXINS

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### INTRODUCTION

The purpose of this chapter is to provide a brief review of the literature on botulinum neurotoxin and tetanus toxin. This review will emphasize studies that pertain to cellular and subcellular actions of the toxins on mammalian preparations, and it will deal exclusively with issues that relate to blockade of exocytosis.

Botulinum neurotoxin and tetanus toxin act on nerve endings and other secretory cells to inhibit spontaneous and evoked mediator release.<sup>1-3</sup> The two toxins are thought to act in a somewhat similar manner, with the exception of intracellular trafficking. During the course of natural poisoning, botulinum neurotoxin binds preferentially to motor nerve endings. It is internalized by the process of receptor-mediated endocytosis, and it escapes endosomes by an acidification process. The toxin then acts locally at motor nerve endings to block acetylcholine release, and this produces flaccid paralysis. The scheme of events with tetanus toxin is somewhat different. This toxin also binds to motor nerves, and it too is internalized by receptor-mediated endocytosis, but it does not act locally. Tetanus toxin is carried by retrograde axonal transport to the central nervous system, where it exits the primary neuron, crosses the synaptic space, and preferentially binds to inhibitory nerve endings. At this point the mechanism of action of tetanus toxin is thought to be similar to that of botulinum neurotoxin. Tetanus toxin is endocytosed and released into the cytosol by an acidification mechanism. By virtue of blocking the release of inhibitory transmitters, it produces disinhibition of excitatory activity. This is believed to be the underlying basis for toxin-induced spastic paralysis.

In addition to its well-known ability to evoke spastic paralysis, tetanus toxin can also produce flaccid paralysis.<sup>2</sup> When large amounts of toxin are administered to laboratory animals, or when high concentrations are added to isolated neuromuscular preparations, some fraction of the toxin escapes retrograde axonal transport and acts locally at nerve endings to block exocytosis. In this case, the general features of botulinum neurotoxin poisoning and tetanus toxin poisoning are essentially the same.

## ORIGIN AND STRUCTURE

Botulinum neurotoxin is produced mainly by *Clostridium botulinum*, but it is also made by *Clostridium baratii* and *Clostridium butyricum*. *Clostridium botulinum* synthesizes seven serotypes of toxin designated A, B, C, D, E, F and G; *Clostridium baratii* synthesizes a single serotype that is similar to type F, and *Clostridium butyricum* synthesizes a single serotype similar to type E. At the moment, *Clostridium tetani* is the only organism known to make tetanus toxin.

Regardless of origin, the synthesis and processing of botulinum neurotoxin and tetanus toxin are identical. Each neurotoxin is synthesized as a single chain polypeptide with a molecular weight of approximately 150,000. In the immediate post-translational stage the neurotoxins have diminished biological activity. When exposed to proteases, the single chain toxins are nicked to yield dichain molecules in which a light chain ( $M_r \sim 50,000$ ) is linked by a disulfide bond to a heavy chain ( $M_r \sim 100,000$ ). This represents the biologically active form of the toxin.

The relationship between nicking and activation has been studied in some detail.<sup>4</sup> The results indicate that nicking is essential but not sufficient for full activation to occur. Several other possible sites for proteolytic cleavage have been considered, including the aminoterminal of the light chain and the aminoterminal of the heavy chain, but these appear not to be related to activation. On the other hand, Giménez, and DasGupta<sup>5</sup> have obtained evidence that suggests that activation includes proteolytic cleavage at the carboxyterminus of the heavy chain.

Most of the clostridial neurotoxins have been sequenced, beginning with tetanus toxin<sup>6,7</sup> and botulinum neurotoxin type A.<sup>8,9</sup> The data indicate that there is significant sequence homology among the various botulinum neurotoxins, and furthermore the botulinum neurotoxins show sequence homology with tetanus toxin. A comparison of botulinum neurotoxin type A and tetanus toxin helps to illustrate the point. An overall comparison shows that there is 30-40% identity between the toxins, and when conservative changes are taken into account the identity rises to 40-50%. The greatest amount of similarity exists in the aminoterminal regions of the heavy chains, which may be the domains that promote internalization (see below).

The work on the primary structures has confirmed a belief that arose from neuropharmacologic research. The various botulinum neurotoxins and tetanus toxin are all descendants of the same ancestral parent. The primary structure work also confirms certain findings in immunology. Monoclonal antibodies have been found that recognize epitopes in several clostridial neurotoxins.<sup>10-12</sup> The commonality of epitopes is an indicator of commonality of structure.

There is much work that remains to be done on the structure of the toxins, and then to relate structure to function, but at least an elementary scheme has already evolved.<sup>13</sup> It appears that the heavy chain, and more precisely the carboxyterminus of the heavy chain, plays an important role in tissue targeting the toxin. The aminoterminal of the heavy chain appears to be essential for the internalization process. The light chain acts inside mammalian cells to block exocytosis, and therefore this portion of the molecule contains the domain that poisons transmission. There is a prevailing belief that the light chain is an enzyme (viz., protease), but the substrate remains unknown.

## BIOLOGICAL ACTIVITY

### Definitions

As an antecedent to discussing the mechanism of action of the toxins, it will be useful to introduce the concepts of universal antagonists and differential antagonists. A universal

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### Universal Antagonist

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antagonist will be defined as a drug or procedure that delays the actions of all serotypes of botulinum neurotoxin and tetanus toxin. A differential antagonist will be defined as a drug or procedure that substantially inhibits the actions of one or several clostridial neurotoxins, but it is not capable of antagonizing all toxins.

The value of universal antagonists is that they help to clarify events that are common to all toxins, such as binding, internalization and escape from endosomes. By contrast, differential antagonists help to reveal unique characteristics of individual toxins.

## Universal Antagonists

**1. Binding.** The receptors for botulinum neurotoxin and tetanus toxin have not been isolated and characterized (but see the promising work reported by Schiavo et al.<sup>14</sup>). However, work from a variety of laboratories on the binding of iodinated toxins to membrane preparations has revealed one interesting point. The various clostridial neurotoxins do not share the same receptor. Each serotype of botulinum neurotoxin appears to have its own receptor, as does tetanus toxin.

In spite of the apparent differences in receptors, there is one characteristic they may have in common. Each receptor may contain a sialic acid residue, or be closely associated with a membrane determinant that has sialic acid residues. This belief stems from earlier work showing that certain classes of gangliosides can cause loss of biological activity of tetanus toxin<sup>15-17</sup> and botulinum neurotoxin.<sup>18,19</sup> This work showed that clostridial neurotoxins had affinity for complex gangliosides, and sialic acid residues were important to the toxin-ganglioside interaction. Free sialic acid was relatively ineffective in producing loss of toxicity.

A more recent and somewhat different line of research tends to support the proposed involvement of sialic acid residues. A large number of lectins with affinity for different sugars were tested as potential antagonists of botulinum neurotoxin and tetanus toxin.<sup>20</sup> Two types of assays were done. In the first, individual lectins were tested for their ability to antagonize the binding of iodinated toxin to rat brain membrane preparations. In the second, these same lectins were tested for their abilities to antagonize the neuromuscular blocking properties of toxin.

Of the various lectins that were tested, two showed promising levels of activity. *Triticum vulgaris* lectin and *Limax flavus* lectin both antagonized the binding of toxins to membranes and the activity of toxins on isolated tissues. The lectin from *Triticum vulgaris* has affinity for N-acetyl- $\beta$ -glucosamine and N-acetyl- $\alpha$ -sialic acid, but the lectin from *Limax flavus* has affinity only for N-acetyl- $\alpha$ -sialic acid. The possible involvement of N-acetyl- $\beta$ -glucosamine as a component of the receptor was ruled out by testing a lectin with affinity for this sugar (*Datura stramonium*).

There were three major points that emerged from the lectin work.<sup>20</sup> First, these compounds were universal antagonists of clostridial neurotoxins. They blocked the activity of all serotypes of botulinum neurotoxin and tetanus toxin in both assays. Second, the lectins were very effective in diminishing the binding of toxins to brain membrane preparations. When tested at adequately high concentrations (e.g.,  $3 \times 10^{-5}$  to  $10^{-4}$  M), they virtually abolished binding. And third, the lectins were very active in diminishing the neuromuscular blocking properties of the toxins. For example, at a concentration of  $3 \times 10^{-3}$  M, the lectin from *Triticum vulgaris* diminished the apparent potency of botulinum neurotoxin type B by nearly two orders of magnitude.

Findings such as these tend to implicate sialic acid residues in the binding of clostridial toxins to nerve membranes. However, they do not clarify the nature of the involvement. A conventional interpretation of the data might be that the receptor is a sialoglycoprotein. Hence, preincubation of membranes with lectins that have affinity for sialic acid could lead to occlusion of the receptor and blockade of toxin binding. A more intriguing interpretation has been advanced by Montecucco.<sup>21</sup> He has proposed that binding may represent a sequence of two events. There could be an initial binding step that involves a charged lipid, such as a ganglioside. This relatively low affinity binding would promote association between toxin and the plasma

membrane. This would facilitate the subsequent high affinity binding of toxin to its authentic receptor. This is a provocative idea that deserves serious consideration. In the meantime, it should be noted that lectins with affinity for sialic acid could also act as antagonists in the sequential binding model.

## 2. Internalization

**A. Plasma Membrane.** Productive internalization of clostridial neurotoxins involves a sequence of events that can be summarized as follows.<sup>13</sup> Toxin molecules bind to membranes of vulnerable cells, after which they are internalized by the process of receptor-mediated endocytosis. This allows toxin to cross the plasma membrane while being retained within an endosome. Passage through the endosome membrane is thought to be initiated by the endosome itself. The membrane possesses a proton pump that progressively lowers pH within the organelle. The toxin molecule possesses a "pH sensor", and when endosomal pH falls to a critical level the sensor triggers a conformational change that exposes a hydrophobic domain that inserts into the endosome membrane. Insertion is the key event in toxin passage across the membrane, although it is not yet known how passage occurs.

The initial work to implicate a membrane penetration step was done on the neuromuscular junction. A series of studies with polyclonal antibody showed that surface-bound toxin disappeared from accessibility to extracellular antibody and that this occurred before onset of paralysis.<sup>1,22</sup> These results suggested that toxin had to be internalized. Subsequent work demonstrated that several drugs known to block receptor-mediated endocytosis were toxin antagonists. The first agent to be tested was chloroquine and its analogs,<sup>23</sup> and this was followed by work with ammonium chloride and methylamine hydrochloride.<sup>24</sup> The results indicated that all three drugs antagonized botulinum neurotoxin, whereas ammonium chloride and methylamine hydrochloride but not chloroquine antagonized tetanus toxin. This work, in combination with the polyclonal antibody studies, was taken as evidence that clostridial toxins were internalized by receptor-mediated endocytosis.

The pharmacological work on internalization was nicely complemented by the morphological work of Black and Dolly,<sup>25,26</sup> who iodinated botulinum neurotoxin and used it to do electronmicroscopic autoradiography at the neuromuscular junction. They were able to show that botulinum neurotoxin binds to the plasma membrane, and membrane-bound toxin could subsequently be localized in endosomes. Movement of toxin across plasma membranes and into endosomes was energy-dependent and accelerated by nerve stimulation. Ammonium chloride, methylamine hydrochloride and chloroquine altered uptake and/or distribution of toxin. In sum, the morphological data were very supportive of the earlier pharmacological data.

**B. Endosome Membrane.** The current belief is that clostridial neurotoxins possess a pH sensor that induces conformational changes that lead to exposure of a hydrophobic domain. This in turn causes the toxins to insert into the endosomal membrane and eventually penetrate it. Several lines of research support this model.

A representative study is the one conducted by Hoch et al.<sup>27</sup> on pH-induced channels in planar lipid bilayers. These workers added botulinum neurotoxin to one side of an artificial membrane, then altered pH on the cis or trans side of the membrane. They observed relatively few channels with an iso-pH of 7.0 or 4.0, but they saw rapid appearance of channels when pH on the cis-side was lowered to approximately 5.5 or below and pH on the trans-side was maintained at 7.0. Structure-function analyses revealed that the heavy chain was responsible for channel formation.

Subsequent work by Donovan and Middlebrook,<sup>28</sup> Blaustein et al.,<sup>29</sup> and Shone et al.<sup>30</sup> confirmed that botulinum neurotoxin formed pH-dependent channels in lipid bilayers. Donovan and Middlebrook<sup>28</sup> demonstrated that toxin-induced channels inserted permanently into the membrane and fluctuated between open and closed states. Blaustein et al.<sup>29</sup> and Shone et al.<sup>30</sup>

extended the structure of the heavy chain.

Biochemical studies of botulinum neurotoxin and its receptor used photoreactive lipids that at acid pH caused the toxin to suggest that it was the result of a hydrophilic ligand that achieved translocation.

Kamata et al.,<sup>3</sup> demonstrated that the cooperativity between the two chains of botulinum neurotoxin is a result of a conformational change that occurs at the same result was observed.

There is a close relationship between the two membranes. Indeed, botulinum neurotoxin and tetanus toxin showed that they caused release of calcium from the endoplasmic reticulum and Rauch et al.<sup>38</sup> demonstrated that they formed channels in membranes.

There is evidence that the hydrophobic domain of botulinum neurotoxin,<sup>39</sup> photoactivated, forms a complex with the aminoterminal half of the heavy chain. Our colleagues have shown that this is the case.

To summarize, the target cells to produce the effect are due to receptor-mediated endocytosis, a mechanism that is well established.

The putative mechanism of action of botulinum neurotoxin is a universal antagonist of acetylcholine release and it possesses the ability to bind preferentially on valine residues. It can be used to inhibit the release of inhibiting ATPase.

Recent studies have shown that all serotypes of botulinum neurotoxin nor did it alter intracellular pH or of productive internalization or acidification of endosomes.

## Differential Antagonism

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extended the structure-function analyses by localizing channel activity to the aminoterminal half of the heavy chain.

Biochemical techniques have also been used to demonstrate that pH can cause botulinum neurotoxin and its respective chains to partition into a lipid environment. Montecucco et al.<sup>31,32</sup> used photoreactive reagents to monitor toxin insertion into the core of a lipid bilayer. They found that acid pH caused both the heavy and light chains to insert and be labelled. This prompted them to suggest that it was not necessary to view the heavy chain as an agent for promoting passage of a hydrophilic light chain into the cytosol. Instead, the two chains could act cooperatively to achieve translocation.

Kamata et al.,<sup>33</sup> who used a fluorescent reporter group, obtained data that support the notion of cooperativity between the two chains. Their work showed that low pH induced striking conformational changes in the intact toxin leading to exposure of hydrophobic domains. The same result was obtained when the heavy and light chains were examined independently.

There is a closely related body of research that pertains to tetanus toxin insertion into membranes. Indeed, investigation of tetanus toxin began before the corresponding studies with botulinum neurotoxin. Boquet and Duflo<sup>34</sup> prepared asolectin vesicles loaded with potassium, and they showed that at low pH tetanus toxin inserted into the membrane and created a pore that caused release of cation. Borochoy-Neori et al.,<sup>35</sup> Gambale and Montal,<sup>36</sup> Menestrina et al.<sup>37</sup> and Rauch et al.<sup>38</sup> have used electrophysiologic techniques to show that tetanus toxin forms channels in membranes.

There is evidence that pH-induced changes in tetanus toxin lead to exposure of a hydrophobic domain. This was demonstrated by measuring [<sup>3</sup>H]-Triton X-100 association with toxin,<sup>39</sup> photoactivatable phospholipid association with toxin,<sup>40</sup> and fluorescent reporter group association with toxin.<sup>33</sup> Each study has demonstrated that the hydrophobic domain is in the aminoterminal half of the heavy chain and the light chain. In a related study, Cabiaux and her colleagues have shown that low pH causes tetanus toxin to induce fusion of lipid vesicles.<sup>41</sup>

To summarize, there is a consensus among workers that clostridial neurotoxins must enter target cells to produce blockade of exocytosis. Crossing of the plasma membrane is probably due to receptor-mediated endocytosis. Crossing of the endosome membrane is due to a mechanism that is triggered by a fall in pH.

The putative role of an acid-triggered mechanism has led to the discovery of another universal antagonist, a compound known as bafilomycin. This compound is microbial in origin, and it possesses the property of inhibiting membrane ATPase. Interestingly, bafilomycin acts preferentially on vacuolar ATPase rather than plasma membrane ATPase. Therefore, bafilomycin can be used to inhibit the ATPase-dependent proton pump in endosomal membranes without inhibiting ATPase-dependent ion pumps in plasma membranes.

Recent studies (Simpson, in preparation) have shown that bafilomycin is an antagonist of all serotypes of botulinum neurotoxin and tetanus toxin. The drug did not inhibit toxin binding nor did it alter intracellular expression of toxicity. Bafilomycin appeared to inhibit the process of productive internalization. This is in keeping with the action of a drug that inhibits acidification of endosomes.

## Differential Antagonists

There are two known groups of differential antagonists, one of which was fully predictable and the other of which emerged quite unexpectedly. Neutralizing antibodies are an example of the former and drugs that promote acetylcholine release, such as the aminopyridines, are an example of the latter.

**1. Antibodies.** Although antibodies have been identified that cross-react with more than one clostridial neurotoxin (see above), no antibody has been found that neutralizes all neurotoxins. Both the linear and conformational characteristics of the active domains within



each serotype are sufficiently unique to preclude the generation of antibodies that bind to active sites in multiple serotypes. Because of this, antibodies have enjoyed little utility as research tools for probing the common structural properties of clostridial neurotoxins.

In spite of the limited value of antibodies in defining common structural elements, they may be helpful in clarifying the sequence of events in poisoning. A study with monoclonal antibodies against botulinum neurotoxin type E helps to illustrate the point.<sup>42</sup> A family of monoclonal antibodies was isolated, and from this family it was possible to identify three that possessed special utility. Each of the antibodies diminished the potency of native toxin, and each interacted with a different domain in the toxin (i.e., the light chain, the aminoterminal of the heavy chain or the carboxyterminal of the heavy chain). Additionally, each of the antibodies recognized a conformational rather than a linear epitope, and it was this property that allowed them to be used as research tools.

Neuromuscular preparations were incubated with toxin under conditions that allowed binding but prevented internalization. These tissues were then exposed to one or more of the monoclonal antibodies. The results showed that monoclonal antibodies were almost as effective in diminishing the potency of bound toxin as they were in diminishing the potency of free toxin. An outcome like this reveals two interesting things about toxin binding to neuronal membranes. First, binding does not cause any of the three domains in the toxin to disappear completely from the cell surface. At least that portion of each domain that possessed the relevant epitope was still exposed. Second, binding may not be associated with major changes in the conformation of the toxin molecule. Each of the antibodies was directed against a conformational epitope, and each had substantial activity against free toxin and bound toxin. At a minimum, this means that the respective epitopes did not undergo major changes during binding.

A larger family of antibodies that recognize all epitopes in the toxin molecule would allow one to determine whether any part of the toxin undergoes conformational changes during binding. In the meantime, the data indicate that binding may be very different from internalization. As explained above, pH-induced internalization is associated with marked changes in conformation.

**2. Aminopyridines.** Drugs such as 4-aminopyridine and 3,4-diaminopyridine block voltage-dependent potassium channels in nerve endings. By virtue of delaying the process of repolarization, these drugs promote influx of calcium and secondarily increase efflux of acetylcholine.

The ability of these drugs to enhance stimulus-evoked release of acetylcholine led to their evaluation as possible antagonists of botulinum neurotoxin. The initial results with serotype A appeared rather favorable. The drugs slowed the rate of onset of toxin-induced paralysis of isolated neuromuscular preparations, and they even reversed - at least temporarily - the development of mild paralysis. However, the results with other serotypes were less promising. Aminopyridines provided little or no protection against most serotypes of botulinum neurotoxin and tetanus toxin.

When the actions of the aminopyridines were studied at the electrophysiological level, the differences between and among serotypes became more obvious. The disparity between responses to type A, on the one hand, and responses to type B and tetanus toxin were especially clear.<sup>43-45</sup> Neuromuscular preparations poisoned with serotype A had a very low rate of spontaneous miniature endplate potentials, and the probability of evoking an endplate potential was also low. However, the addition of aminopyridines substantially increased the likelihood of evoking an endplate response. By contrast, neuromuscular preparations poisoned with botulinum neurotoxin type B or tetanus toxin showed a different result. Following addition of aminopyridines, only rarely was there an evoked endplate potential.

Closer examination of poisoned tissues appeared to reveal a reason for the disparate responses. In the case of serotype A, the combination of nerve stimulation and aminopyridines resulted in synchronous release of quanta, and thus the increased likelihood of observing an

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One especially interesting finding arose from dual poisoning experiments.<sup>46</sup> Tissues exposed initially to serotype A displayed synchronous release, but when they were subsequently exposed to serotype B or tetanus toxin the release became desynchronized. When toxins were added in the reverse order, release was always desynchronized. These data with aminopyridines, as well as other drugs, toxins and physical procedures, led to the hypothesis that there could be two sites of clostridial neurotoxin action.<sup>46</sup> Botulinum neurotoxin type A was postulated to act at a site somewhat remote from the membrane, whereas serotype B and tetanus toxin acted closer to the membrane, and hence closer to the site of exocytosis.

## INTRACELLULAR ACTIONS

There is compelling evidence to show that clostridial neurotoxins act in the cell interior to block exocytosis. Over the period of approximately a decade there has been a progression of research to demonstrate this point. The work began with pharmacologic experiments involving the use of antibody escape and the use of drugs known to antagonize endocytosis.<sup>22,23,24</sup> This was followed by studies on electronmicroscopic localization of toxin at various stages during poisoning.<sup>25,26</sup> Next, it was shown that direct intracellular injection of toxin blocked exocytosis.<sup>47</sup> This prompted experiments in which toxin, or polypeptides derived from toxin, were introduced into cells by permeabilization techniques.<sup>48-51</sup> Most recently, it has been shown that intracellular injection of mRNA that encodes toxin leads to expression of toxin and blockade of transmitter release.<sup>52</sup>

In addition to demonstrating that clostridial toxins act in the cell interior, this work has highlighted an anomaly for which there is currently no explanation. When tested in mammalian preparations, the light chains of clostridial neurotoxins are both necessary and sufficient to block exocytosis. By contrast, when tested in *Aplysia*, the light chains are necessary but not sufficient.<sup>53</sup> There is a domain in the heavy chain that is needed in addition to the light chain to produce poisoning of transmitter release. There are other structure-function differences between toxin action on mammalian preparations and *Aplysia*, and these differences have yet to be explained.

## Messenger Systems

The fact that the toxins act inside nerve terminals has encouraged investigators to search for possible targets. There has been a tendency to divide these possible targets into four broad categories: i) messenger systems, ii) the cytoskeleton, iii) vesicles, and iv) the plasma membrane. Relatively little work has been done with the specific intent of evaluating vesicles or membranes as sites of toxin action. However, there is an emerging literature on messenger systems and cytoskeleton.

Several laboratories have evaluated the possibility that clostridial neurotoxins modify messenger systems that govern transmitter release. This effort has been largely negative, but one possible exception is the work on protein kinase C.

Considine and his colleagues have demonstrated that agonist-induced changes in protein kinase C activity in NG-108 cells are inhibited by tetanus toxin. This has been shown both for an artificial agonist (e.g., phorbol ester<sup>54</sup>) and for a natural agonist (e.g., neurotensin<sup>55</sup>). This work raises the possibility that protein kinase C may be a target for clostridial neurotoxins. This possibility was tested by determining the effects of known inhibitors of protein kinase C on transmitter release at the mammalian neuromuscular junction (Considine, Sherwin and Simpson, in preparation). Two inhibitors of the catalytic domain (H7, staurosporine) and two

inhibitors of the regulatory domain (calphostin, sphingosine) were added individually to phrenic nerve-hemidiaphragm preparations at concentrations that virtually abolished protein kinase C activity in neuronal cell cultures (e.g., NG-108). Interestingly, the tissues continued to respond for several hours even under conditions in which the enzyme was substantially or completely inhibited. Furthermore, pretreatment of tissues with inhibitors of protein kinase did not predispose them to the poisoning effects of clostridial neurotoxins.

The results with protein kinase C inhibitors give rise to three conclusions. First, the enzyme is not required to sustain short-term neuromuscular transmission. Second, protein kinase C cannot be the target for clostridial toxins. And third, protein kinase C cannot be the target for any toxin that acts rapidly to block neuromuscular transmission.

### Cytoskeleton

A number of hypotheses have been advanced that implicate the cytoskeleton, and especially the actin-based cytoskeleton, in the process of transmitter release. One of the more thoroughly studied of these models is one that pertains to mediator release from adrenal chromaffin cells. According to this model, it is envisioned that storage granules are held in place by a lattice-work of actin filaments. When an agonist acts on the cell to trigger mediator release, one of its effects is to stimulate disaggregation of actin lattices. This releases storage granules that can then move toward the plasma membrane and discharge their contents.

It is unclear whether a similar model applies to cholinergic nerve endings. However, if the cytoskeleton does play a role in exocytosis, it might conceivably be the target for clostridial neurotoxins. Therefore, a series of studies have been undertaken to evaluate the possible role of actin and related molecules in cell poisoning. One line of research has focused on actin and the cholinergic neuromuscular junction<sup>56</sup> (Considine and Simpson, unpublished findings); a second line of research has concentrated on microtubules and central nervous system synaptosomes.<sup>57</sup>

The role of actin in exocytosis and toxin action has been evaluated by using the botulinum binary toxin as a research tool.<sup>58</sup> This toxin is composed of two separate and independent polypeptide chains (Mr ~45,000 and 100,000), both of which are necessary to produce cell poisoning. The heavy chain is a tissue targeting domain that binds to receptors on vulnerable cells. The heavy and light chains of the binary toxin have no affinity for one another in solution, but when the heavy chain associates with membranes of vulnerable cells it creates a docking site for the light chain. The heavy and light chains are then internalized by the process of receptor-mediated endocytosis.<sup>59</sup>

The light chain of the botulinum binary toxin is an enzyme that possesses mono(ADP-ribosyl)transferase activity.<sup>60</sup> The substrate for the enzyme is monomeric actin.<sup>61</sup> By virtue of modifying G-actin, the binary toxin disrupts the cytoskeleton of cells. More precisely, the binary toxin exerts two specific effects. Monomeric actin is in dynamic equilibrium with filamentous or F-actin. When the binary toxin ADP-ribosylates G-actin, it irreversibly removes these molecules from the pool of monomeric actin and this in turn promotes dissociation of filamentous actin to maintain equilibrium. A related effect is that ADP-ribosylated actin acts as a capping protein, thus preventing elongation by unmodified actin.

The consequences of binary toxin action are easy to observe in cultured cells. The toxin causes flattened and varigated cells that are attached to the plating surface to retract and become round.<sup>62-64</sup> In essence, the cells collapse on themselves because they lack a cytoskeleton.

The botulinum binary toxin has been used to study exocytosis in a variety of secreting cells, and the results do not encourage a belief that the actin-based cytoskeleton plays either a simple or a universal role in mediator release. For example, treatment of neuromuscular preparations with the binary toxin has no obvious effect on stimulus-evoked transmitter release. Similarly, pretreatment of cells with the binary toxin neither enhances nor inhibits the actions of clostridial neurotoxins.<sup>56</sup> A survey of binary toxin action on all secreting cells that have been studied to

date reveals no consistent mediator release, inhibiting another. These results c

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date reveals no consistent action on either spontaneous or evoked mediator release.<sup>58</sup> Enhanced mediator release, inhibited mediator release or no effect has been observed in one cell type or another. These results do not indicate a link between actin and clostridial neurotoxins.

Another line of inquiry has been directed at microtubules. Dolly and his colleagues have examined the ability of microtubule dissociating drugs to alter the actions of clostridial neurotoxins on norepinephrine release from rat brain synaptosomes.<sup>57</sup> They have observed that these drugs have little or no effect on certain toxins (e.g., serotype A), but they exert a small and statistically significant effect on others (e.g., serotype B). Interestingly, this disparity of action is the same as that observed with differential antagonists such as aminopyridines, but in the opposite direction. Aminopyridines antagonize serotype A but have little action on serotype B and tetanus toxin.

It is encouraging to see that two lines of research (aminopyridines and other differential antagonists; microtubule dissociating drugs) are coming to the same apparent conclusion. There may be two classes of clostridial neurotoxins, with serotype A being a prototype of one class and serotype B and tetanus toxin being prototypes of the other. Hopefully, the molecular basis for this division will be discovered shortly.

## Enzymatic Actions

Although the specific target for clostridial neurotoxins has not been identified, there is reason to suspect that the toxins are enzymes with protease activity. Work on the primary structures of the toxins has revealed that they contain a histidine motif that is characteristic of zinc-containing proteases.<sup>8</sup> This observation has prompted several laboratories to undertake a vigorous search for a substrate. Some tantalizing preliminary observations were presented during the conference, but no authentic substrates for toxin action were identified.

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